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No Association Between Schizophrenia and the Serotonin Receptor 5HTR2a in an Italian Population

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A major role of the serotonergic system has been hypothesized in the pathogenesis of schizophrenia, mostly based on the evidence of action of new and atypical neuroleptics such as Risperidone or Clozapine. We evaluated the genotypes and alleles of the 5HT2a receptor gene in 67 nuclear families following the Haplotype Relative Risk (HRR) strategy and in a second sample of 100 schizophrenics and 103 controls. The 5HT2a receptor gene polymorphism, following PCR amplification and subsequent Hpa II digestion, reveals a two-alleles system in the coding region of the gene. We did not find statistically significant differences between patients and controls for genotypes, nor for alleles, both in the HRR and in the case-control groups. These results do not confirm the positive association obtained by Inayama et al.: [Neuropsychopharmacology 1(35):145–219, 1994] and by Williams et al. [Lancet, 397:1294–1296, 1996] in our population. *Am. J. Med. Genet. 74:21–25, 1997.*

KEY WORDS: association; HRR; 5HTR2a; psychiatric genetics; schizophrenia

INTRODUCTION

Schizophrenia is a severe mental disorder associated with disturbances of thought, affect, volition, and behavior, characterized by highly variable age of onset and different degrees of functioning and intellectual deterioration. Data from twin, adoption, and family studies have demonstrated clearly that genetics plays an important role in the etiology of the disease.

Despite the lack of unequivocal knowledge about the hypothesized genetic transmission of schizophrenia, there has been a recently growing effort to find the molecular basis of this disease. Of special interest is the interaction between dopaminergic and serotonergic systems with evidence, from pharmacological data in animals, that each of these systems may exert an inhibitory influence on the other. Antagonist of both dopamine and serotonin receptors have been developed knowing that dopaminergic and serotonergic systems in the mammalian central nervous system functionally interact [Leysen et al., 1993]. Thus balanced antagonism of these receptors may provide an enhanced antipsychotic effect. Drugs such as Risperidone and Clozapine, which show a more incisive pharmacological action and a novel receptor binding profile than the classic antipsychotic drugs, may improve symptoms in otherwise resistant illness, or where intolerance prevents the use of standard treatments [Arranz et al., 1995; Masellis et al., 1995].

The serotonin hypothesis emerged in the 1950s following observations of the striking similarities between serotonin and the hallucinogen LSD. Later, reports that serotonergic-depleting drugs such as Reserpine can alleviate some symptoms of schizophrenia led to the theory that an increase in serotonin may be causally related to the disease [Stahl and Wets, 1987]. Furthermore, serotonin has become of much interest in schizophrenia research since the observation that many of the so-called atypical antipsychotics have potent serotonin-related activities.

Specifically, antagonism at the serotonin type 2a (5HT2a) receptor has been emphasized as important in reducing psychotic symptoms and in mitigating against the development of D2-antagonism-related movement disorders: D2 receptor blockade seems to be essential for the treatment of positive symptoms of schizophrenia, but is responsible for extrapyramidal side effects (EPS), whereas the predominant 5HT2a receptor blockade may reduce the EPS liability and can ameliorate negative symptoms of schizophrenia [Kapur and Remington, 1996].

More evidence of the interest of serotonin in schizophrenia comes from the finding of Joyce et al. [1993] that serotonin uptake sites and receptors are altered in

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the limbic system of schizophrenics. Moreover, studying 5HT2a receptor binding, the maximum number of binding sites (Bmax) was significantly decreased in schizophrenic patients as compared to normal controls. This difference did not appear to be due to neuroleptic treatment [Arora et al., 1991].

Thus hypothesizing a possible dysfunction of the 5HT neurotransmitter system in schizophrenia, a Hinc II polymorphism for the human serotonin 1d receptor variant (5HT1d beta) was first tested by Sidenberg et al. [1993] for linkage to schizophrenia in five Canadian pedigrees. Although one of the five pedigrees tested had a slightly positive Lod-score, there was no overall evidence for linkage to schizophrenia under dominant, recessive, or two locus models. Also, Hallmayer et al. [1992], using multipoint linkage analysis between schizophrenia and genetic markers spanning the region of the 5HTR2a locus, excluded linkage between this candidate gene and schizophrenia in a Swedish kindred. The 5HTR2a gene is located on chromosome 13, and a new *Msp*I polymorphism for this gene has been noted by Warren et al. [1994], consisting of a T/C polymorphism at position 102 of the 5HTR2a gene. Both allele 1 (TCT) and allele 2 (TCC) encode for a Serine at aminoacid 34. Digestion of the 372 bp PCR product with *Msp*I (or the isoschizomer *Hpa*II) yields a 372 bp product for allele 1 and 156 and 216 bp for allele 2 [Warren et al., 1994].

Inayama et al. [1994] found a positive association between genotype 2/2 and allele 2 of the 5HTR2a and schizophrenia using a classical case-control study. A recent report by Williams et al. [1996] confirms this finding on a large data set collect through a multicenter collaboration. Overall, across seven population samples pooled together, 5HT2a is associated to schizophrenia with an odds ratio of 1.3; if analyzed separately, only two samples over seven showed a significant Chi-square for association. Thus, 5HT2a might be a "minor" gene in conferring susceptibility to schizophrenia. Aiming to replicate these previous findings, we evaluated the 5HTR2a genotypes in a first sample consisting of 67 nuclear families following the Haplotype Relative Risk strategy, and in a second sample of 100 unrelated schizophrenic patients and 103 controls in a classical case control study.*

We also typed the DRD4 gene in 52 of the 67 nuclear families suitable for the Haplotype Relative Risk to evaluate the possibility of an interaction between the two markers and the disease. Interest in evaluating this potential interaction is based on our findings that DRD4 showed a robust association with psychotic symptomatology in a very large set of psychiatric patients (Serretti et al., sub.).

MATERIALS AND METHODS

Sample

After obtaining informed consent, both parents of 67 additional schizophrenic patients were recruited in order to apply the HRR strategy. Both the affected subjects and the parents were native Italians, all from the same areas of Northern and Central Italy. As second sample, following a classical case control study, we investigated 100 schizophrenics (60 males and 40 females) recruited from hospitalized patients at the Department of Neuropsychiatric Science of the S. Raffaele Hospital in Milan and 103 healthy controls (51 males and 52 females).

All the patients were administered the Diagnostic Interview Schedule, revised version (DIS-R) [Robins et al., 1989; Battaglia et al., 1995], and all were diagnosed according to DSM-IIIR criteria. The controls were both staff members of S. Raffaele Hospital and healthy subjects attending the general hospital laboratories for screening blood testing and randomly selected from the general population. Each control subject had been given at least a general medical screening for major illnesses. All participants in the study were native to Northern Italy. The mean age of patients was 50.7 (sd: 16.7), mean age of onset 23.1 (sd: 7.5), whereas for controls mean age was 38.1 (sd: 12.1).

DNA Analysis

Venous blood samples, anticoagulated with EDTA, were drawn from all patients and parents. DNA was then extracted as described in Lahiri and Nurnberg [1991].

We typed the 5HTR2a gene using a 9600 Perkin Elmer Cetus thermocycler: a polymerase chain reaction (PCR) was carried out with the following primers: 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCC GCC GTC TGC TAC AAG TTC TGG CTT- 3′ and 5′-CTG CAG CTT TTT CTC TAG GG-3′. Genomic DNA (100 ng) was diluted to 12.5 μl using water and heated to 99°C for 3 min. Then, a reaction mixture was added containing 1.5 U/sample Taq Polymerase, 1X Taq Polymerase Buffer, 0.5 mM of each primer, 100 mM of dATP, dCTP, dTTP, dGTP in a total volume of 25 μl. Thirty cycles were performed with a profile of 95°C for 20 sec, 60°C for 20 sec, and 72°C for 20 sec. This profile was followed by a 72°C chase for 4 min. The PCR products were then digested with the HpaII restriction enzyme for 2 hr, and subsequently the digestion products were analyzed in 3% agarose gel. This polymorphism reveals a 2 allele system.

For the D4 typing, the methods have been described in detail elsewhere [Lichter et al., 1993; Macciardi et al., 1994a,b]. This PCR polymorphism is particularly complex and detects a 8 alleles system (A2 = 0.10, A3 = 0.03, A4 = 0.7, A5 = 0.06, A6 = 0.03, A7 = 0.08, A8 = 0.03, A10 = 0.03; where 2–10 corresponds to the number of 48bp repeats).

Statistical Analysis

We used the experimental design described by Terwilliger and Ott [1992] for HRR to test for deviations from linkage disequilibrium, considering the genotype of the affected offspring as the "case" and the genotype made up of the alleles not transmitted to the child from its parents as the "control" sample in an association test. The same data structure collected for HRR is also useful for the Transmission Disequilibrium Test (T.D.T) [Spielman et al., 1993; Baur, 1994], which

*This sample is part of the EMASS: European Multi-Centre Association Study of the Molecular Genetics of Schizophrenia [Williams et al., 1996].

investigates the hypothesis of association under the assumption that the two genes (i.e., the marker/candidate and the susceptibility genes) are linked and in linkage disequilibrium. In this case the appropriate statistical analysis is the Mc Nemar's test.

RESULTS

Haplotype Relative Risk Sample

Up to now, 67 nuclear families for 5HTR2a have been typed. A standard Chi-square test does not reveal any statistically significant association between schizophrenia and the 5HTR2a genotypes using the Haplotype Relative Risk design (Table I).

Following the hypothesis that an allele, more than a genotype, could be responsible for a putative association with the disease, we also explored this hypothesis, but again, we did not find any association between 5HTR2a and schizophrenia (Table II).

As expected, given the not significant HRR, also the TDT test for 5HT2a showed not significant results (Data not shown.) Fifty-two of the 67 nuclear families suitable for the HRR have also been typed for DRD4. Since the different DRD4 alleles also may show different biochemical properties, we focused our attention on three different alleles (2,4,7), evaluating the hypothesis that any of these alleles could be associated with one of the 5HTR2a alleles in the affected subjects. No statistically significant association has been found with any alleles of the 5HTR2a polymorphism stratified by coding polymorphism at locus DRD4 (Table III).

Case-Control Sample

To complement the HRR design, we report here results from our case-control sample [Williams et al., 1996] showing absence of significant association. A standard Chi-square test does not reveal any statistically significant association between the disease and the 5HTR2a genotypes (Table IV) or alleles (Table V) in our Italian population.

DISCUSSION

Both Serotonin and Dopamine have been implicated in a large number of psychophysiologic processes. Serotonergic neurotransmission represents a complex mechanism involving pre- and postsynaptic events and distinct serotonin receptor subtypes. Serotonin (5HT) receptors have been classified into several categories, and they have been termed 5HT1, 5HT2, 5HT3, 5HT4, 5HT5, 5HT6, and 5HT7 receptors. Serotonin-1 receptors have been further subdivided into 5HT1a, 5HT1b, 5HT1c, 5HT1d, 5HT1e, and 5HT1f. Serotonin-2 receptors have been divided into 5HT2a, 5HT2b, and 5HT2c receptors. All 5HT2 receptor subtypes are linked to the multifunctional phosphoinositide (PI) signaling system. Serotonin-3 receptors are considered ion-gated receptors and are also linked to the PI signaling system by an unknown mechanism [Pandey et al., 1995]. Whereas the roles of the 5HT2c and 5HT3 receptors are unclear, the 5HT2a receptor subtype is the most widely studied of the 5HT receptors in psychiatric disorders as well as in relation to the mechanism of action of neuroleptic and antidepressant drugs.

Disturbances of serotonergic pathways have been implicated in a wide variety of neuropsychiatric disorders such as depression, anxiety, migraine, schizophrenia, alcoholism, aggression, suicidal behavior, Tourette's syndrome, and substance abuse. Moreover, the 5HT2 receptor has been implicated in a number of behavioral and physiological processes, and it also may play a role in cellular development and differentiation and may represent a site of action of hallucinogens and certain psychotherapeutic drugs [Morilak et al., 1994]. Genetic variation in genes coding for serotonin receptor proteins could be considered a mechanisms for the genetic

TABLE I. Distribution of Genotipic Frequencies for 5HTR2a and Schizophrenia Following HRR Strategy

	1	2	Total
Transmitted	49	18	67
Not-transmitted	51	16	67
	100	34	134

Chi-square = 0.16 df: 1 p: ns.

TABLE II. Distribution of Allele Frequencies for 5HTR2a and Schizophrenia Following HRR Strategy of Sampling

	1	2	Total
Transmitted	65 (48.5%)	69 (51.5%)	134
Not transmitted	72 (53.7%)	62 (46.3%)	134
	137	131	268

Chi-square = 0.73 df: 1 p: ns.

TABLE III. Distribution of Allelic Frequencies of the 5HTR2a and DRD4 in Nuclear Families

DRD4 allele 2

5HTR2a	1	2	Total
Transmitted	12 (50%)	12 (50%)	24
Not transmitted	18 (75%)	6 (25%)	24
	30	18	48

Chi-square = 2.22 df: 1 p: 0.136.

DRD4 allele 4

5HTR2a	1	2	Total
Transmitted	54 (45.8%)	64 (54.2%)	118
Not transmitted	60 (50.8%)	58 (49.2%)	118
	114	122	236

Chi-square = 0.42 df: 1 p: 0.516.

DRD4 allele 7

5HTR2a	1	2	Total
Transmitted	12 (42.9%)	16 (54.1%)	28
Not transmitted	9 (32.1%)	19 (67.9%)	28
	21	35	56

Chi-square = 0.30 df: 1 p: 0.583.

TABLE IV. Distribution of Genotypic Frequencies for 5HTR2a and Schizophrenia

Genotypes	11	12	22	Total
Affected	18 (18%)	52 (52%)	30 (30%)	100
Controls	27 (26.2%)	48 (46.6%)	28 (27.2%)	103
	45	100	58	203

Chi-square = 1.99 df: 2 p: 0.369.

susceptibility to these diseases, as well as an issue of pharmacogenetic relevance. Inayama et al. [1994] reported that the frequency of genotype 2-2 and the allele 2 of 5HTR2a was significantly higher in schizophrenics than in controls in a Japanese population. Actually, in a well-selected group of schizophrenic patients collected in different countries across Europe, allele 2 of 5HT2a showed a significant association, despite conferring a weak risk for the disease [Williams et al., 1996].

Given other evidence of 5HT2a being related with response to clozapine treatment, Arranz et al. [1995] found that homozygosity for the c102 allele was more frequent (53%) among patients who did not respond to clozapine than in those who responded (25%). This finding could be considered as evidence that allelic variation of genes that encode neurotransmitter receptors can influence clinical response to antipsychotic drugs. Thus it is still possible that this gene could be considered as a "minor gene" in conferring susceptibility to schizophrenia; however, it is still also possible that the interaction with other genes is more crucial. Under this perspective, we evaluated a complex polymorphism on the exon III of DRD4 gene that shows a strong association with psychiatric symptomatology in a very large set of Italian patients and that is of great importance as a putative site of action for typical and atypical neuroleptics, but neither the interaction with a Dopamine receptor (D4) seems to be of some relevance.

In conclusion, our study does not provide evidence of any association between schizophrenia and genotypes or alleles of the 5HT2a gene, in two different samples using also a powerful design strategy like the Haplotype Relative Risk. Therefore, although population genetic differences between Caucasian and not Caucasian populations can account for these controversial results, our finding seems to exclude a main involvement of the 5HT2 gene in the etiopathogenesis of schizophrenia, at least in our population.

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TABLE V. Distribution of Allelic Frequencies for 5HTR2a and Schizophrenia

Alleles	1	2	Total
Affected	88 (44%)	112 (56%)	200
Controls	102 (44.5%)	104 (50.5%)	206
	190	216	406

Chi-square = 1.24 df: 1 p: 0.265.

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